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Haplotype–Phenotype Correlation in Fukuyama Congenital Muscular Dystrophy

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In typical Fukuyama congenital muscular dystrophy (FCMD), peak motor function is usually only unassisted sitting or sliding on the buttocks, though a few patients are able to walk at some point. However, a few patients have a severe phenotype and never acquire head control. In addition, it is clinically difficult to differentiate this severe FCMD from Walker-Warburg syndrome (WWS) or from muscle–eye–brain disease (MEBD). In order to establish a genotype–phenotype correlation, we performed haplotype analysis using microsatellite markers closest to the FCMD gene (*FCMD*) in 56 Japanese FCMD families, including 35 families whose children were diagnosed as FCMD with the typical phenotype, 12 families with a mild phenotype, and 9 families with a severe phenotype. Of the 12 propositi with the mild phenotype, 8 could walk and the other 4 could stand with support; 10 cases were homozygous for the ancestral founder (A-F) haplotype whereas the other 2 were heterozygous for the haplotype. In the 9 severe cases, who had never acquired head control or the ability to sit without support, 3 had progressive hydrocephalus, 2 required a shunt operation, and 7 had ophthalmological abnormalities. Haplotype analysis showed that 8 of the 9 cases of the severe phenotype are heterozygous for the A-F haplotype, and the other one homozygous for the haplotype. We confirmed that at least one chromosome in each of the 56 FCMD patients has the A-F haplotype. The rate of heterozygosity for the A-F haplotypes was significantly higher in severe cases than in typical or mild cases ($P < 0.005$). Severe FCMD patients appeared to be compound heterozygotes for the founder mutation and another mutation. Thus, the present study yielded molecular genetic evidence of a broad clinical spectrum in FCMD. Am. J. Med. Genet. 92:184–190, 2000.

KEY WORDS: genotype–phenotype correlation; clinical severity; ancestral founder haplotype; compound heterozygosity

INTRODUCTION

Congenital muscular dystrophy (CMD) with central nervous system involvement comprises three clinical variants: Fukuyama CMD (FCMD) [Fukuyama et al., 1960], Walker-Warburg syndrome (WWS) [Dobyns et al., 1985; Dobyns et al., 1989], and muscle–eye–brain disease (MEBD) [Raitta et al., 1978; Santavuori and Leisti, 1977]. These entities share clinical manifestations, and the distinction between FCMD and the other two may depend largely on a quantitative difference in severity [Fukuyama, 1997]. FCMD is one of the most common autosomal recessive disorders in the Japanese population whereas WWS and MEBD are seldom diagnosed in Japan. The characteristic brain malformation of these three disorders is cobblestone lissencephaly with cerebral and cerebellar cortical dysplasia due to a defect in neuronal migration [Takada et al., 1984]. The clinical severity of brain and eye involvement in

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FCMD is milder than in WWS and MEBD. Clinically, peak motor function is usually only unassisted sitting or sliding on the buttocks although a few patients are able to walk at some point [Fukuyama et al., 1981]. Our previous linkage analysis showed that the ambulatory cases belong to a clinical spectrum of FCMD [Kondo-Iida, Saito, Osawa et al., 1997; Kondo-Iida, Saito, Tanaka et al., 1997]. However, there are a few patients who have a severe phenotype and never acquire head control, and it is clinically difficult to differentiate severely affected FCMD cases from WWS.

Recently, molecular genetic analyses were conducted in an effort to elucidate the pathogenesis of FCMD. Since 1993, when the FCMD gene (*FCMD*) was mapped to chromosome area 9q31–33 [Toda et al., 1993], new microsatellites flanking the gene have been developed [Miyake et al., 1997; Toda et al., 1994; Toda et al., 1996]. Based on haplotype analysis using markers closest to the gene, most *FCMD*-bearing chromosomes are derived from a single ancestral founder [Kobayashi et al., 1998a]. *FCMD* has been cloned and the gene product was named fukutin [Kobayashi et al., 1998b]. A 3-kb retrotransposal insertion was detected as the ancestral founder mutation of *FCMD*. These advances have facilitated the diagnosis of FCMD [Kondo-Iida et al., 1997a; Kondo-Iida et al., 1997b; Nakano et al., 1996; Saito et al., 1998; Yamamoto et al., 1996; Yamamoto et al., 1997a; Yamamoto et al., 1997b].

The purpose of this study is to establish a phenotype–genotype correlation in FCMD. We describe the result of haplotype analysis of FCMD chromosomes among 56 Japanese FCMD families using markers closest to the FCMD gene.

MATERIALS AND METHODS

Subjects

In this study, clinical phenotypes of FCMD were classified into the following three groups according to the patients' maximum motor abilities: (a) The typical phenotype is assigned to patients who were able to sit unassisted or to slide on the buttocks (levels 2–4) [Okawa and Ueda, 1997], (b) A mild phenotype is defined if patients could stand or walk with or without support (levels 5–8), and (c) a severe phenotype is defined if patients were able to sit only with support or had no head control (levels 0–1).

Thirty-five patients with the typical FCMD phenotype, 12 with the mild phenotype, and 9 with the severe phenotype participated in this study. All 56 patients came from unrelated families. Three patients with the mild phenotype are able to go up and down stairs (level 8), 5 are able to walk alone (level 7), and 3 are able to stand with support (level 5) (Table I) whereas in the severe cases, 7 were bedridden and lacked head control (level 0) and 2 acquired head control but were unable to sit without support (level 1) (Table II). All cases were clinically evaluated and the diagnosis was confirmed by a biopsy done by one or more of the authors. Dystrophic muscle changes and gross brain malformation (gyral abnormalities) were demonstrated.

The clinical findings in some of the mild cases were described previously [Kondo-Iida et al., 1997b]. Brain MRI revealed gyral abnormalities of a milder degree in all 8 mild cases examined, mild ventricular delatation in 7 (88%) but no brain stem hypoplasia (Table I, Fig. 1a). Ocular abnormalities observed included fundus abnormality in 1 (11%) of 9, strabismus in 1 (8%) of 12, and myopia in 4 (44%) of 9 patients. Of the 9 severe cases, 2 (Cases 13 and 14) had progressive hydrocephalus, and both required a shunt operation (Table II). One patient (Case 15) showed progressive ventriculomegaly and is now under close observation; shunt placement is being considered. One patient (Case 17) died of acute pneumonia at age 1$\frac{3}{12}$ years. All 9 patients examined by MRI or CT scan showed severe gyral abnormality and ventricular dilatation, and all 8 cases examined by MRI showed brain stem hypoplasia (Table II, Fig. 1b). Seven patients had ocular abnormalities: retinal hypoplasia in 5 (56%), strabismus in 5 (56%), optic nerve atrophy in 3, and macular hypoplasia, myopia, and choroid atrophy in 1 each. Most notably, Case 18 had severe ocular anomalies (microphthalmia, retinal detachment, retinal hypoplasia, and

TABLE I. Motor, Brain, and Ocular Status of Mild Cases of FCMD*

			Motor	Brain			Eye			
No.	Sex	Age at last observation	Maximum ability	Gyral abnormality	Ventricular dilatation	Brain stem hypoplasia	Fundus abnormality	Strabismus	Myopia	Others
1	F	16:0	8	ND	ND	ND	–	+	–	–
2	F	20:1	8	+	+	–	–	–	+	–
3	F	20:10	8	+	+	–	–	–	+	Cataract
4	M	10:11	7	+	–	–	+	–	–	–
5	F	15:11	7	ND	ND	ND	ND	–	ND	ND
6	F	17:3	7	+	+	–	–	–	–	Nystagmus
7	F	23:6	7	ND	ND	ND	ND	–	ND	ND
8	M	27:7	7	+	+	–	–	–	–	–
9	M	7:6	5	+	+	–	–	–	+	–
10	M	10:5	5	+	+	–	–	–	–	–
11	F	22:7	5	ND	ND	ND	ND	–	ND	ND
12	F	27:10	5	+	+	–	–	–	+	–
Positive rate				8/8	7/8	0/8	1/9	1/12	4/9	2/9
(%)				100	88	0	11	8	44	22

*Maximum motor ability: 5 = maintain stand posture with support, 7 = ambulant, 8 = walk up stairs; ND, not determined.

TABLE II. Motor, Brain, and Ocular Status of Severe Cases of FCMD*

			Motor	Brain				Eye		
No.	Sex	Age at last observation	Maximum ability	Gyral abnormality	Ventricular dilatation	Hydrocephalus	Brain stem hypoplasia	Fundus abnormality	Strabismus	Ant. chamber abnormality
13	M	8:2	1	++	++	+ ope	+	+	+	–
14	M	4:4	0	++	++	+ ope	+	–	–	–
15	M	1:10	0	++	++	+	+	–	–	–
16	M	1:2	0	++	++	–	+	–	+	–
17	F	1:3	0	++	++	–	NC	+	+	–
18	M	0:4	0	++	++	–	+	++	–	+
19	F	1:3	0	++	++	–	+	–	+	–
20	F	3:5	0	++	++	–	+	+	+	–
21	M	11:8	1	++	++	–	+	+	–	–
Positive rate				9/9	9/9	3/9	8/8	5/9	5/9	1/9
(%)				100	100	33	100	56	56	11

*Maximum motor ability: 0 = not acquired head control, 1 = acquired head control but unable to sit without support; NC, not confirmed; ope, shunt operation.

anterior chamber abnormalities) regarded as hallmarks of WWS or MEBD. This patient also had mild tetralogy of Fallot and a normal 46,XY karyotype.

Haplotyping (Allelotyping)

DNA was extracted from peripheral blood leukocytes of the patients, their parents, and sibs. In Family 5, DNA was obtained from a paraffin-embedded muscle specimen from the propositus [Savois et al., 1997] because she was deceased. All subjects provided informed consent to undergo the haplotype analysis.

We used the following microsatellite markers: D9S2105, D9S2107 [Toda et al., 1996], and D9S172 [Dib et al., 1996] as well as D9S306 (*mfd 220*), which was previously mapped to chromosome 9q31 [Toda et al., 1994]. In addition, we used D9S2170 and D9S2171 [Kobayashi et al., 1998a], which were further isolated between D9S2105 and D9S2107 with the strongest linkage disequilibrium with FCMD [Toda et al., 1996]. The FCMD gene (*FCMD*) is located very closely (centromerically) to D9S2170. The order of these markers was as follows: cen-D9S306-D9S2105-FCMD-D9S2170-D9S2171-D9S2107-D9S172-tel; the distance between D9S2105 and D9S2107 was approximately 230 kb. Primer sets for polymerase chain reaction (PCR) amplification of these markers were synthesized. Conditions for PCR and subsequent electrophoresis were as described previously [Saito et al., 1998; Toda et al., 1994; Toda et al., 1996; Toda et al., 1993]. PCR was performed in a 25 μl reaction mixture containing 20 ng of genomic DNA, 20 pmol of one unlabeled primer, and 20 pmol of one primer end-labeled with 1.0 mCi of [γ-^{32}P]ATP using T4 polynucleotide kinase, ×1 PCR buffer (16.6 m*M* NH_4SO_4, 67 m*M* Tris-HCl, pH 8.8, 10 m*M* β-mercaptoethanol, 6.7 μM EDTA), 10% (v/v) dimethyl sulfoxide, 1.5 m*M* of each dNTP, 5 m*M* $MgCl_2$, and 1.25 U Taq DNA polymerase. For D9S306, samples were incubated in a DNA thermocycler (Perkin Elmer Cetus, Norwalk, CT) for 35 cycles under the following conditions: 94°C for 1.5 min, 55°C for 2 min, and 72°C for 1.5 min. The first denaturation and final elongation steps were extended to 3 min and 10 min, respectively. The PCR products were analyzed on 6% polyacrylamide gel and visualized by autoradiography.

Statistical Analysis of FCMD Severity Versus Allelotyping

The χ^2 statistical analysis was performed in 25 patients with the typical phenotype, 12 with the mild phenotype, and 9 with the severe phenotype in order to clarify whether FCMD severity is determined by homozygosity for the ancestral founder (A-F) haplotype or compound heterozygosity.

RESULTS

Haplotype analysis for the six markers revealed that 27 (77%) of 35 patients with the typical phenotype have the A-F haplotype (138–192–147–183–301 for D9S2105-D9S2170-D9S2171-D9S2107-D9S172) on both FCMD-bearing chromosomes, 7 (20%) have this haplotype on one chromosome, and 1 (3%) is a homozygote for another haplotype (146–196–147–193–293) different from the A-F haplotype (Table III).

Of the 12 patients with the mild phenotype, 10 were homozygous for the A-F haplotype whereas the other 2 (Families 4 and 7) were heterozygous for this haplotype (Table III, Fig. 2). In 1 patient, we could not obtain any PCR products for D9S172 from genomic DNA of the paraffin-embedded muscle specimen. This patient probably had a historical recombination between D9S2171 and D9S2107, leading to a haplotype of 138–192–147–193 for D9S2105-D9S2170-D9S2171-D9S2107, on one A-F haplotype-bearing chromosome.

Eight patients with the severe phenotype are heterozygous for the A-F haplotype (Table III, Fig. 3) whereas 1 patient (Family 21) is homozygous for the haplotype but with a historical recombination (138–192–147–183–297) on one chromosome (Fig. 3). In 5 patients (Families 13–17) including 3 patients with hydrocephalus (Families 13–15), the other chromosome that does not bear FCMD showed a 130–201–157–183–295 haplotype (Fig. 3). Likewise, a 158–201–151–197–297 haplotype was detected in 2 (Families 18 and 19) and a 130–201–161–183–297 haplotype in 1 (Family 20). The patient in Family 18 with various ocular anomalies and that in Family 19 with only strabismus (Table II) showed the same haplotypes on each chromosome.

As a result, 27 (77%) patients with the typical phenotype and 10 (83%) with the mild phenotype were

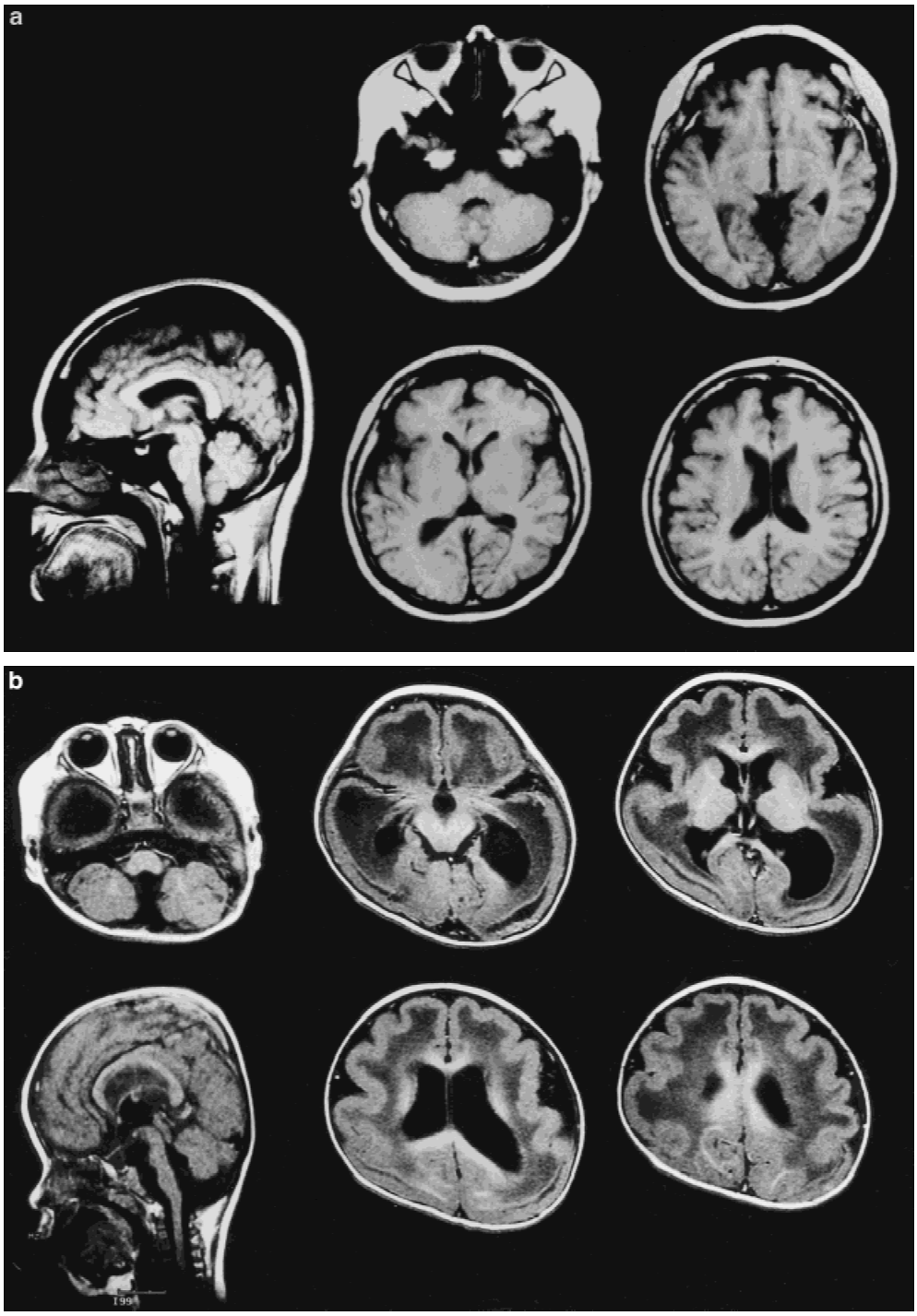

Fig. 1. Shown are the brain MRI (T1-weighted image) of FCMD patients. Gyral abnormalities of a milder degree and mild ventricular delatation but no brain stem hypoplasia in a patient (Case 2) with the mild phenotype **(a)**, and severe gyral abnormality with double cortex in occipital area, ventricular dilatation, and brain stem hypoplasia in a patient (Case 16) with the severe phenotype **(b)**.

homozygous for the A-F haplotype whereas 8 patients (89%) with the severe phenotype are heterozygotes (Table IV). The homozygosity rate, that is, the rate of having the A-F haplotype on both chromosomes, was significantly higher ($P < 0.005$) in typical cases and mild cases than in severe cases. In other words, the heterozygosity rate was significantly higher ($P < 0.005$) in severe cases than in typical and mild cases.

DISCUSSION

The FCMD-associated A-F haplotype-bearing chromosomes, as demonstrated by the three closest markers (D9S2105, D9S2107, and D9S172), were present in 60 (77%) of 78 chromosomes in our previous report [Saito et al., 1998] whereas Toda et al. [1996] obtained a rate of 75%. Recently, two new closest microsatellites

TABLE III. Haplotypes in a Chromosome Other Than the Chromosome That Bears the Founder Haplotype in FCMD Patients and Their Parents*

Haplotypes at 5 marker loci D9S2105-S2170-S2171-S2107-S172	Number of chromosomes in FCMD patients with phenotypes			Number of control chromosomes in parents
	Typical	Mild	Severe	
138–192–147–183–301[a]	27[b]	10[b]	1[b]	1[b]
138–192–149–193–301	2			
138–201–155–183–297	4	1		
138–201–153–183–297	1			
138–201–157–183–297		1		1
130–201–157–183–295[c]			5	
130–201–161–183–297			1	
158–201–151–197–297			2	
146–196–147–193–293	2 (1)[d]			1
Other haplotypes				79
Total	36 (35)	12	9	82

[a]The founder haplotype.
[b]Homozygote for the founder haplotype.
[c]Second founder haplotype.
[d]Homozygote for the 146–196–147–193–293 haplotype.
*The number in parentheses is number of patients.

(D9S2170 and D9S2171) were developed, and a founder haplotype was represented as 138–192–147–183 for D9S2105-D9S2170-D9S2171-D9S2107, which was shared by 119 (82%) of the 145 FCMD chromosomes [Kobayashi et al., 1998a]. In this study, where these two new microsatellite markers (D9S2170 and D9S2171) were added, FCMD patients had at least one A-F haplotype-bearing chromosome, except for 1 typical case who is homozygous for a different haplotype (146–196–147–193–293) (Table III). Furthermore, 79 normal chromosomes from parents of FCMD patients had haplotypes not observed on FCMD-bearing chromosomes. Interestingly, the A-F haplotype was observed in one such normal chromosome, suggesting that it bears the wild-type *FCMD* allele without mutation. The founder mutation is assumed to have been introduced into the Japanese population 100 generations (2,000–2,500 years) ago [Kobayashi et al.,1998a]. As there was apparently high consanguinity at that time, FCMD carriers with a single *FCMD* mutation have spread throughout Japan. At present, the carrier frequency is assumed to be about 1% in the Japanese population [Fukuyama and Ohsawa, 1984; Osawa, 1978]. Thus, the detection of the A-F haplotype is valuable in diagnosing FCMD patients.

When the FCMD haplotypes other than the founder haplotype were compared (Table III), 8 haplotypes were obtained. As *FCMD* lies just centromeric to D9S2170 [Kobayashi et al., 1998a], a historical recombination occurred between D9S2170 and D9S2171 to

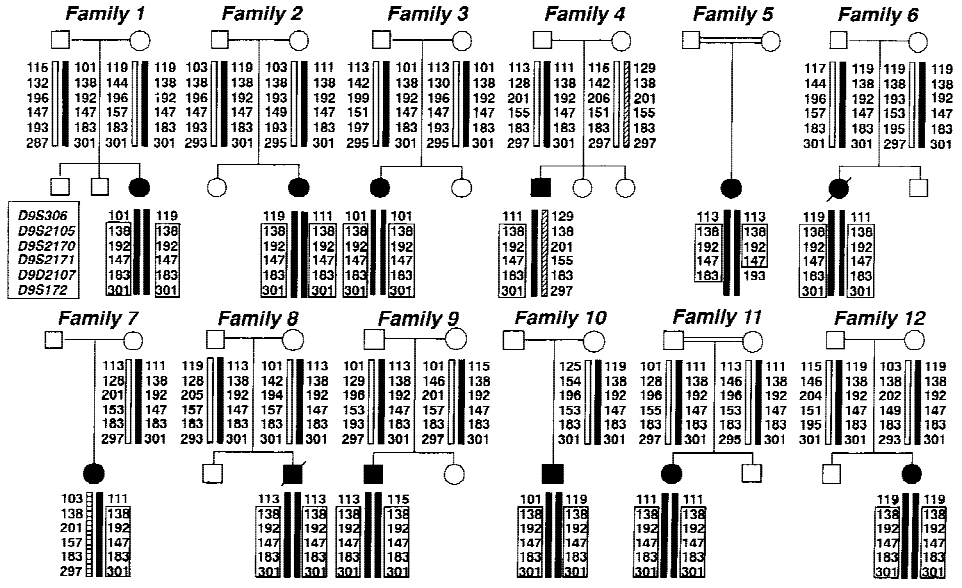

Fig. 2. Six-locus haplotype from members of 12 pedigrees of mild FCMD cases are shown. In Families 1, 2, 3, 5, 6, 8, 9, 10, 11, and 12, the probands have the ancestral founder haplotype (highlighted with square) and are homozygous whereas the other 2 (Families 4 and 7) were heterozygous for the same haplotype.

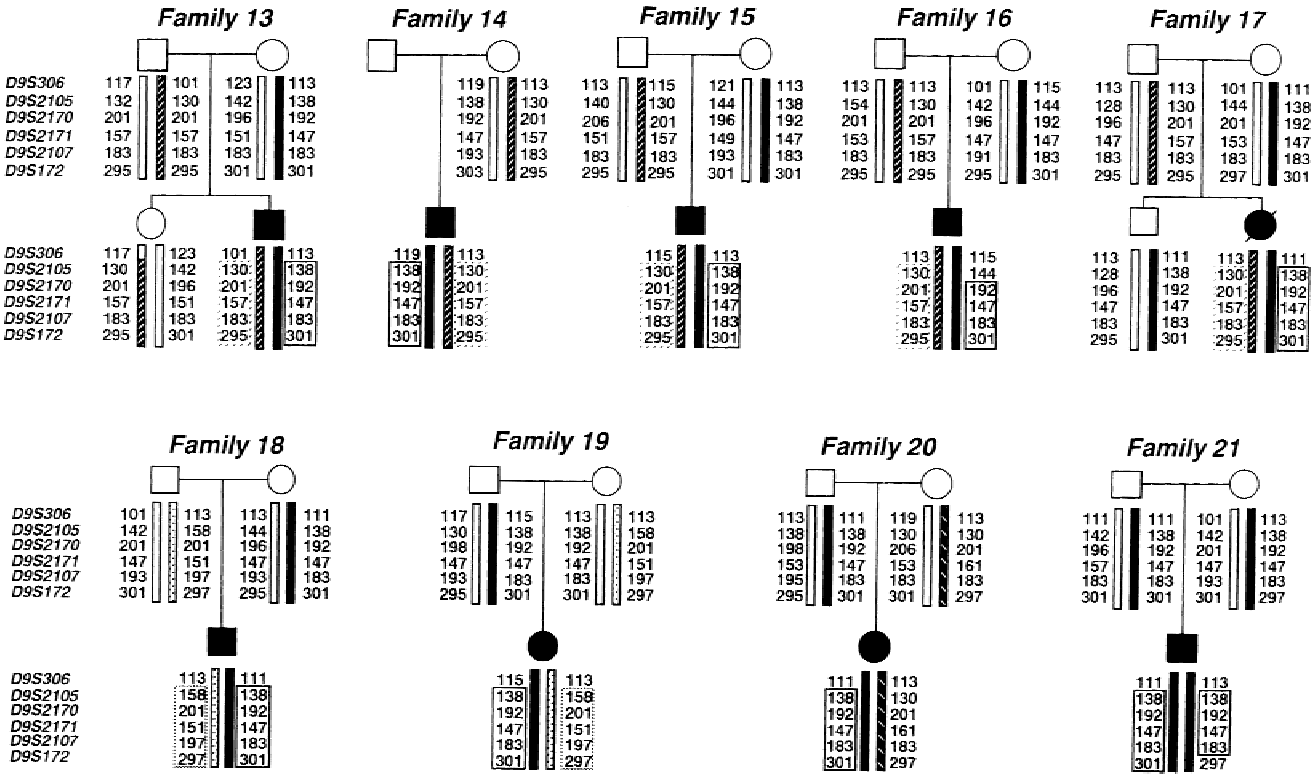

Fig. 3. Six-locus haplotypes from members of 9 pedigrees of severe FCMD cases are shown. The probands in Families 13, 14, 15, 16, 17, 18, 19, and 20 are heterozygous for the ancestral founder haplotype whereas the proband in Family 21 is homozygous but with a historical recombination on one chromosome. The other chromosome showed a 130–202–157–183–295 haplotype in 5 patients (Families 13, 14, 15, 16, and 17), a 158–202–151–197–297 haplotype in 2 patients (Families 18 and 19), and a 130–202–161–183–297 haplotype in 1 patient (Family 20).

make the 138–192–149–193–301 haplotype, which originated from the A-F haplotype. The three haplotypes, 138–201–155–183–297, 138–201–153–183–297, and 138–201–157–183–297, are inferred to originate from the same ancestor who carried a mutation other than the founder mutation. The 130–201–157–183–295 haplotype can be regarded as a second founder haplotype as suggested by Kobayashi et al. [1998a] because it is second most predominant. The 130–201–161–183–297 haplotype is presumed to be a historical recombinant of the second founder haplotype. Interestingly, these haplotypes were not observed in typical and mild FCMD cases.

WWS is an extreme form of CMD, with the most severe brain malformation, eye involvement, and a very short life span. It is characterized by type II lissencephaly with progressive hydrocephalus, agyria, and ocular abnormalities [Dobyns et al., 1985; Dobyns et al., 1989]. MEBD is another rare disorder characterized by severe mental retardation, hypotonia, and visual failure. As there are many common clinicopathological findings between FCMD, WWS, and MEBD, a lingering question has arisen on whether these diseases are allelic. Clinical severity, especially on the brain and ophthalmological involvement in FCMD, is milder than in WWS and MEBD [Dobyns et al., 1985; Fukuyama, 1997]. Chromosomal location of the WWS locus remains unknown whereas the MEBD locus has recently been mapped to 1p32–34 [Cormand et al., 1999]. The present haplotype analysis showed that all 9 severe cases examined have the A-F haplotype in either or both of FCMD-bearing chromosomes: Eight are heterozygotes and 1 is homozygote. Therefore, it is most likely that patients with the severe phenotype defined in these patients lie at one end of the FCMD clinical spectrum and are distinct from WWS or MEBD even though they had severe brain and ophthalmological involvement reminiscent of WWS. The variable phenotypes, that is, mild, typical, and severe phenotypes in FCMD patients, may be due to environmental effects, the existence of modifier genes, or allelic heterogeneity.

TABLE IV. Chi-Square Statistical Analyses Between FCMD Phenotypes and Haplotyes*

	Haplotype			
Phenotype	Homozygosity	Heterozygosity	Others	Probability
Typical	27 (77%)	7 (20%)	1 (3%)	NS
Mild	10 (83%)	2 (17%)	0	$P < .005$
Severe	1 (11%)	8 (89%)	0	$P < .005$

*Others, homozygosity for haplotypes other than the ancestral ones; NS, not significant.

Generally, in autosomal recessive disorders, homozygous mutations may lead to severe clinical symptoms because of absent or reduced gene products whereas compound heterozygosity may result in a milder phenotype. However, this rule does not apply to FCMD. Our statistical analysis showed that 77% of typical cases and 83% of mild cases are homozygous for the A-F haplotype whereas 89% of severe cases showed heterozygosity (Table IV). Recently, a 3-kb retrotransposonal insertion at the 3' untranslated region was identified as an A-F mutation (probably as an *FCMD* mutation on the A-F haplotype) [Kobayashi et al., 1998b]. In view of the nature of the founder mutation and the relationship between the clinical severity and zygosity, the insertional mutation may be compatible with survival whereas other mutations in severe cases may be nonsense mutations. Patients homozygous for such a nonsense mutation would be lethal in utero. On the other hand, it is presumed that mutations other than the A-F mutation in typical and mild cases may be silent or missense. This is why the homozygosity and compound heterozygosity for the A-F haplotype are frequently observed in mild, typical, and severe FCMD cases, respectively. This hypothesis is supported by the finding in Case 13 with the severe phenotype, in whom the retroposon-insertional mutation and the other mutation (a nonsense mutation in exon 3 of *FCMD*), that is, compound heterozygosity, was detected [Kobayashi et al., 1998b]. Further analysis on the expressed level of fukutin will clarify this finding.

In conclusion, we confirmed the FCMD-associated A-F haplotype in at least one chromosome from each of the FCMD patients examined. Haplotype analysis yielded molecular genetic evidence of a broad FCMD clinical spectrum. Most patients with the typical and mild phenotypes are homozygous for the A-F haplotype whereas most patients with the severe phenotype may be compound heterozygotes for the A-F haplotype and another haplotype with another mutation.

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